

Osteoarthritis and Cartilage (2009) 17, 798–804

© 2008 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

doi:10.1016/j.joca.2008.11.013

Osteoarthritis and Cartilage

**International
Cartilage
Repair
Society**

Intra-articular injections of hyaluronan solutions of different elastoviscosity reduce nociceptive nerve activity in a model of osteoarthritic knee joint of the guinea pig

A. Gomis*, A. Miralles, R. F. Schmidt and C. Belmonte

*Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-CSIC,**San Juan de Alicante 03550, Spain*

Summary

Objective: To study in guinea pigs knee joints the effects of intra-articular injection of HYADD® 4-G (Fidia—Farmaceutici), a novel hyaluronan (HA)-derived elastoviscous material and of Hyalgan® (Fidia—Farmaceutici), a HA product with very low viscoelasticity, on movement-evoked nociceptor impulse activity from normal and inflamed knee joints.

Design: Nociceptor impulse activity was recorded from single Aδ and C fibers of the medial articular nerve either under control conditions or after induction of an experimental knee joint osteoarthritis (OA) by partial medial meniscectomy and transection of the anterior cruciate ligament (PMM—TACL). The stimuli consisted of standardized innocuous and noxious inward and outward rotations of the tibia against the femur of 50 s duration, repeated every 5 min for 1.5 h.

Results: The number of movement-evoked impulses was significantly augmented 1 day and 1 week after PMM—TACL compared with intact knee joint. The enhanced impulse response to joint movements 1 week following surgery was attenuated by repeated intra-articular injection of HYADD® 4-G and even more prominently by Hyalgan®.

Conclusions: HA products have a reducing action on joint nociceptor discharges that appears to depend predominantly on their role as an elastoviscous filter associated with their rheological properties, but also on a chemical effect on sensitized nociceptive terminals of inflamed joint tissues, possibly linked to the HA concentration.

© 2008 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Key words: Osteoarthritis, Hyaluronan, Elastoviscous solution, Pain, Nociceptors, Animal models.

Introduction

Hyaluronan (sodium hyaluronate, HA) is a glycosaminoglycan present in joint tissues containing nerve terminals, i.e., synovial tissue and capsule, and also in the surface layer of the cartilage and in synovial fluid. This highly elastoviscous polymer fills the intercellular space between the collagen fibrillar network, surrounding all of the cells, the blood and lymph vessels and the neural elements in the fibrous tissue of the joint^{1–3}.

In arthritic conditions, elastoviscosity of the synovial fluid and tissue matrices is abnormally low^{2,3}. Thus, elastoviscous solutions of HA with variable molecular mass (0.5–6 million) have been injected intra-articularly, with the aim of restoring the rheological homeostasis of the joint in osteoarthritis (OA)^{3–5,7}. This treatment was called viscosupplementation^{3,5}. Early human and equine clinical studies showed that with this treatment, the elastoviscosity of the synovial fluid returned to normal levels and simultaneously, the pain associated with the movement of the joint decreased or was completely eliminated^{6,8,9}.

It has been hypothesized that intra-articular HA application causes analgesia by increasing the elastoviscosity of the intercellular matrix of soft tissues around the joint, thus providing a rheological buffer that reduces the force transmitted during joint movements to the transducing membrane elements at sensory nerve terminals innervating the synovial lining and capsule. This proposal was supported by experimental studies showing that intra-articular injection of high elastoviscous HA solutions produced a reduction of movement-induced nerve impulse activity in single nociceptor fibres of normal and acutely inflamed knee joints of cats, rats and guinea pigs^{10–14}. Molecular mass of HA solutions seems to play an important role in this effect because injection of non-elastoviscous solutions of low molecular mass did not cause a decrease of pain, nor of nociceptor activity in experimental animals^{7,10,12}.

The recording from single fine articular afferents of the knee joint while applying knee joint movements of defined torque¹⁵, opened up the possibility to evaluate quantitatively the analgesic effects of HA preparations of various compositions on normal joints or on joints of animals subjected to partial medial meniscectomy (PMM) and transection of the anterior cruciate ligament (TACL), a condition that has been claimed to mimic an osteoarthritic condition. Consecutive to this surgery, joint inflammation was present as evidenced by an increase in knee joint diameter, possibly reflecting an excess of volume of synovial fluid (exudate) that persisted for 3 weeks. In parallel, an augmented nerve

*Address correspondence and reprint requests to: Dr A. Gomis, Instituto de Neurociencias de Alicante, Universidad Miguel Hernández-CSIC, San Juan de Alicante 03550, Spain. Tel: 34-965919597; Fax: 34-965919549; E-mail: agomis@umh.es

Received 5 June 2008; revision accepted 20 November 2008.

response to knee joint movement was observed 1 day and 1 week after surgery¹³. These responses were reduced by Hylan G-F20 a highly viscous Hyaluronic acid preparation. Likewise, a decrease of pain symptoms has been reported after intra-articular injections of hyaluronic acid of different rheology in osteoarthritic patients^{16–20}.

In the present study, the PMM–TACL model of experimentally-induced knee joint OA in guinea pigs was used to evaluate the effect on movement-evoked impulse activity of single knee joint nociceptive nerve fibers, of a newly developed HA product of high elastoviscosity HYADD®4-G²¹, comparing its analgesic effects with those of Hyalgan®, a low elastoviscosity solution of higher HA concentration but similar molecular weight.

Material and methods

The study was performed on 117 guinea pigs of both sexes with ages between 5 and 6 weeks at the time of surgery, weighing 400–450 g. In 50 animals more than one unit could be studied in the same experiment while in the remaining 67 animals, one single fiber per experiment was recorded. Animals were treated in accordance with institutional animal care guidelines.

PMM AND TACL SURGERY

PMM plus TACL were performed in the right hind limb, as a model to induce joint inflammation, and joint instability leading to OA lesions^{22–29}. In brief, animals were anesthetized with an intramuscular injection of a mixture of ketamine (50 mg/kg) and xilacine (10 mg/kg). Anesthesia was maintained using isoflurane (1–1.5%) mixed with 0.5 l of oxygen administered through the respiratory circuit. An antero-medial approach to the joint was achieved via a skin incision extending from the level of the distal third of the femur to the proximal fourth of the tibia. The skin was retracted and the joint capsule opened. The medial meniscus was identified and transected halfway between its anterior and posterior horns. The anterior cruciate ligament was transected afterwards. The joint capsule and the overlying fascia were subsequently sutured.

Sham operated animals were treated identically except that transections of the meniscus and of the anterior cruciate ligament were omitted.

To accelerate the recovery of the animals, they received an injection of 5 mg/kg atipamezol (a xilacine reversing agent). For 3 days after surgery, subcutaneous injections of buprenorphine HCl (an analgesic acting at CNS level) and enrofloxacin (an antibiotic) were applied per day. The knee joint circumference was routinely measured before surgery and at the onset of the experimental procedures for the recordings from the nerve filaments of the various experimental groups. The animals were maintained in the animal house for variable periods of time (from 24 h up to 1 week) prior to the nerve recording session. During this time they were allowed to move freely, drinking and eating ad libitum.

RECORDING PROCEDURES

The animals were anaesthetized initially with an intramuscular injection of a mixture of Ketamine (50 mg/kg) and Xilacine (10 mg/kg). Supplementary doses of anesthetic were injected i.v. as required. Animals were paralyzed by the i.v. administration of 5 mg/100 g gallamine triethiodide i.v. (Sigma) and artificially ventilated. Body temperature was kept around 37°C with a servo-regulated heating pad.

All recordings were done on the right hindlimb. The surgical preparation followed the routine pattern worked out in this laboratory over the last years^{11–13,15}. A tracheotomy was performed. Cannulations of the left femoral vein and artery were also made to apply substances and to control blood pressure. An additional fine catheter was placed into the right saphenous artery distal to the knee joint for close retrograde i.e., application of KCl into the joint area.

Stabilization of the right femur was accomplished by fixation with a special grip, and a pool was formed using skin flaps which was filled with warm paraffin oil. In this pool the whole medial part of the leg was exposed, including the knee joint and the patellar ligament. A shoe-like holder for the hind paw was connected to a lever via a metal rod equipped with a strain gauge and a torque-meter (Hottinger Baldwin Messtechnik, Germany) to measure the applied torque when performing inward and outward rotations of the calf against the thigh.

The recording techniques were analogous to those used in previously^{12,13}. In brief, the saphenous nerve was cut both in the inguinal region and distally from the joint, leaving only the medial articular nerve intact. Fine filaments were dissected at the proximal end of the nerve. After putting the

filaments over a silver wire electrode for extracellular recording, the receptive fields of the afferent units were explored by recording the nerve impulse responses to mechanical stimuli evoked by probing the tissue over the knee joint and its surroundings with a hand-held glass rod.

The afferent terminal of the nerve fiber so identified was electrically stimulated (5–15 V, 0.5 ms) within its receptive field to calculate the conduction velocity from the latency of the evoked impulse and the distance between the stimulating and recording electrodes. Nerve fibers with conduction velocities (CV) <2.5 m/s were classified as C fibers, those with CV from 2.5 to 20 m/s as Aδ fibers¹³.

In order to ensure that the units under observation were being reached by the blood supply to the medial aspects of the knee joint and remained functional, their discharges in response to a close intra-arterial bolus injection of KCl (0.2 ml, 0.3 M, via the catheter in the A. saph., see above) were observed at the end of the recording period.

STIMULATING PROCEDURES

The mechano-sensitivity to knee joint movements of all articular afferent units identified in this way was explored using a movement protocol (see¹²), starting from the middle (the normal resting) position of the joint. It consisted of a passive, manually performed outward rotation within the working range of the joint of 10 s duration (10–20 mNm, innocuous outward rotation, [OR]) followed by an equally long outward rotation which exceeded the normal working range of the joint (40–65 mNm, noxious outward rotation, [NOR]). Thereafter, the joint was returned to the resting position for 10 s and the same two steps were performed using innocuous and noxious inward rotations [IR and NIR]. Such a complete movement cycle ([OR, NOR] followed by [IR, NIR]) with a total duration of 50 s was repeated every 5 min (see plots in Figs. 1–3). The rotation called innocuous occurs in the normal working range of the knee joint; it is not made against resistance and it evokes a very discrete or no activity in joint afferent units^{15,30}.

EXPERIMENTAL DESIGN

The following eight experimental groups were made, according to the treatment received (see also Table I)

- Group 1. Healthy animals.
- Group 2. Healthy animals that received one intra-articular injection of saline solution 1 day before nerve recording.
- Group 3. Healthy animals that received one intra-articular injection of HYADD®4-G 1 day before nerve recording.
- Group 4. Animals with sham surgery performed 1 day and 1 week before nerve recording.
- Group 5. Animals with PMM–TACL surgery performed 1 day and 1 week before nerve recording.
- Group 6. Animals with PMM–TACL surgery together with immediate intra-articular injection of HYADD®4-G recorded 1 day after surgery, and animals with PMM–TACL surgery that received two intra-articular injections of HYADD®4-G, one at the end of the PMM–TACL surgery and one 3 days later (at day 4, i.e., at the third postoperative day). Articular nerve fiber recording was performed 1 week after surgery.
- Group 7. Animals with PMM–TACL surgery that received 2 intra-articular injections of saline solution, one at the end of the PMM–TACL surgery and one 3 days later. Articular nerve fiber recording was performed 1 week after surgery.
- Group 8. Animals that received 2 intra-articular injections of Hyalgan® solution, one at the end of the PMM–TACL surgery and the second, 3 days later. Articular nerve fiber recording was performed at day 7 after surgery.

TEST MATERIAL(S) AND THEIR APPLICATION

Two HA products were used: Hyalgan® and HYADD®4-G. Hyalgan® is a 10 mg/ml solution of 500–730 kDa average molecular weight hyaluronic acid. HYADD®4-G, is the hexadecylamide of hyaluronic acid of 500–730 kDa average molecular weight (5 mg/ml), obtained through chemical modification of 500–730 kDa average molecular weight hyaluronic acid by adding hexadecylamine (a primary aliphatic amine with 16 carbon atoms) to the carboxylic group of glucuronic acid. According to the manufacturing company FIDIA–Farmaceutici, the degree of modification is very low (2% mol/mol), that means that in HYADD®4-G, of every 100 (disaccharide) units of hyaluronic acid (N-acetyl glucosamine and D-glucuronic acid) only 2 have D-glucuronic acid chemically modified by the covalent bound to the hexadecylamine. FIDIA claims that this chemical modification determines a relevant increase of the viscoelastic properties in comparison to the starting Hyalgan® and that at 2.5 Hz the elastic modulus of 5 mg/ml HYADD®4-G is about 50 Pa while the elastic modulus of Hyalgan® is 0.6 Pa.

In this study, Hyalgan® was used as available in the market, therefore at 10 mg/ml in phosphate buffered saline (PBS), while HYADD®4-G was used at a concentration of 5 mg/ml in PBS. 100 µl of the test material and of the

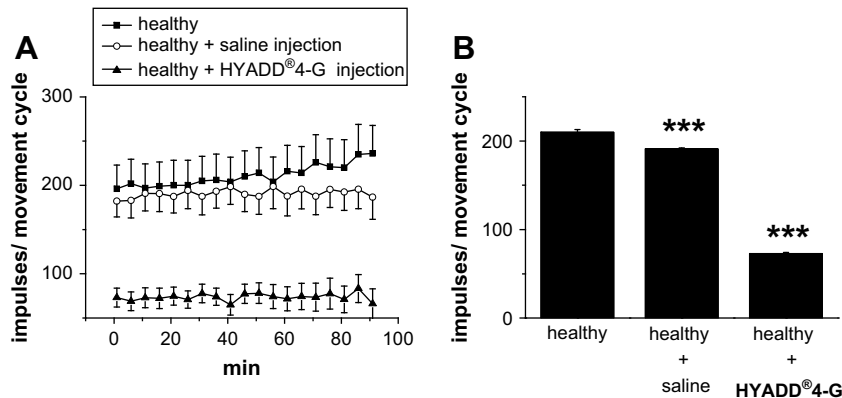


Fig. 1. Movement-evoked activity in healthy knee joint afferents and in afferents 24 h after saline or HYADD®4-G injection into the articular cavity. (A) *Squares*: average number of impulses recorded from primary afferent units innervating healthy knee joints ($n = 18$); *circles*: those of units from healthy knee joints which 1 day before recording received an intra-articular injection of 100 μ l of saline ($n = 10$); and the *triangles* are from units of joints which one day before recording received an intra-articular injection of 100 μ l of HYADD®4-G ($n = 10$). Each point plotted shows the average \pm S.E.M. of the total number of impulses obtained in single primary fine afferent units during a complete movement cycle i.e., OR + NOR + IR + NIR, each cycle starting 5 min after the previous one and lasting 50 s (see *Methods* for a detailed description of a movement cycle) obtained. (B) The bar histograms illustrate the comparison between the three experimental conditions. The ** denote the significance for the treated animals vs control (One-way ANOVA; Kruskal–Wallis test $P < 0.001$).

control saline solution were injected into the joint space from the lateral side using a 26 G needle. This was followed by passive knee joint movements to facilitate the intra-articular distribution of the solution.

ACTIVITY MEASUREMENT

The discharges recorded during the movements were analyzed by counting separately the number of impulses evoked by each of the four movements OR + NOR + IR + NIR. The regular observation was that the change of the nerve activity, depending on the treatment group, occurred both during movements performed within the working range and in the noxious range. For this reason, we used the discharges recorded during the complete movement cycle for presentation of the results. Plots and graphs displayed in the figures were averaged from at least eight individual experiments (for details see legends). The numbers of fibers recorded in each experimental condition is indicated in *Table I*.

STATISTICS

For statistical evaluation, the total number of impulses obtained during the complete cycle was used to represent the response to each individual movement. They were then pooled to find the mean \pm S.E.M. representing the average response of each experimental group. Comparison between mean values of the different experimental groups of animals was performed using the One-way ANOVA. Individual values of knee joint circumferences before and after surgery were compared using the Student's paired t test. The significance level was set at $P < 0.05$ (*).

Results

GENERAL FINDINGS

Successful recordings of appropriate duration (>90 min) were obtained from 174 fine afferent nerve fibers of the median articular nerve (MAN) of 117 animals belonging to the various experimental groups. Most of the MAN nerve fibers of the guinea pig responding to movements of the knee joint had CV that included them either into the A δ (CV = 2.5–20 m/s, fine myelinated fibers) or the C fiber group (CV = <2.5 m/s, unmyelinated fibers). Unequivocal determination of the CV was possible for 62 A δ (mean CV = 3.8 ± 0.2 m/s) and 68 C fibers (mean CV = 1.64 ± 0.1 m/s). The number of C and A δ fibers was variable among groups making difficult in some cases statistical comparisons. Moreover, observed changes in nerve activity with treatment occurred equally in A δ and C fibers.

Therefore data from A δ and C fibers have been presented jointly in the results.

EFFECT OF HYADD®4-G ON MOVEMENT-EVOKED ACTIVITY IN HEALTHY KNEE JOINTS

The solid square symbols in *Fig. 1(A)* represent the average activity of 18 single units from healthy animals evoked by innocuous (20 mN) and noxious (65 mN) outward and inward rotations. Each point represents the average number of impulses evoked by a complete movement cycle that had a total duration of 50 s.

The filled triangles symbols in *Fig. 1(A)* show the movement-evoked impulse activity measured in recordings performed in 10 afferent fibers, 24 h after intra-articular injection of 100 μ l of HYADD®4-G while open circles represent the average, movement-evoked impulse discharge in 10 additional fibers measured 24 h after injection of 100 μ l saline. To perform the injections into the joint cavity the animals were briefly sedated by an intramuscular injection of ketamine. The bar histogram in *Fig. 1(B)* illustrates the summarized results of these experiments, and show that both types of intra-articular injections reduced in a significant way the average discharges of the primary afferent units evoked by knee rotation. However, the injections of HYADD®4-G led to a much more pronounced discharge decline than that of the saline application. Data are summarized in *Table I*.

MOVEMENT-EVOKED ACTIVITY IN PRIMARY KNEE JOINT AFFERENTS AFTER SHAM – AND PMM–TACL – OPERATIONS

The open squares in *Fig. 2(A)* illustrate the data obtained from 11 single fine articular units after PMM–TACL surgery performed one day before the beginning of the electrophysiological recording. The black squares in this plot display the results of 12 similarly conducted experiments except that sham surgery was performed, (for details see *Methods*). Obviously the PMM–TACL surgery led to a significant increase (24% on average) in the evoked afferent outflow from the fine articular afferents (*Table I*).

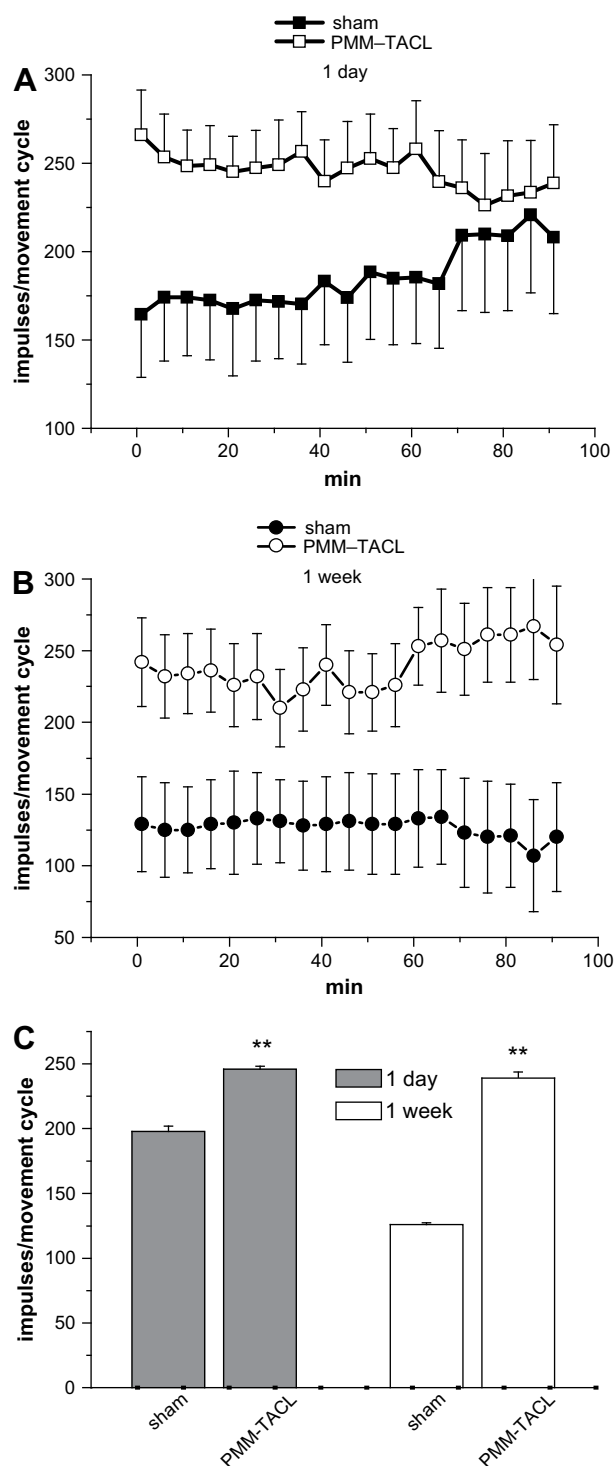


Fig. 2. Movement-evoked activity in primary knee joint afferents after sham and PMM-TAC surgery. Stimulating, recording, evaluating and plotting as in Fig. 1. (A) Average movement-evoked activity in sham (solid squares; $n = 12$) and in PMM-TACL (open squares; $n = 11$) operated animals recorded 1 day after surgery. (B) Average movement-evoked activity in sham (solid circles; $n = 9$) and in PMM-TACL (open circles; $n = 15$) operated animals recorded 1 week after surgery. (C) Summary of the averages of impulses/movement cycle in the indicated experimental conditions. The ** denote the significance for the PMM-TACL animals vs sham animals (One-way ANOVA; Dunn's methods; $P < 0.001$).

Figure 2(B) illustrates a set of similar experiments conducted 1 week after the surgical procedures. 1 week after PMM-TACL surgery there is, in comparison to the outflow from the sham operated joints, still a considerable increase in the evoked impulse outflow from the fine articular afferents (Table I). Results of these 2 sets of experiments are summarized in the grey and white bar histograms of Fig. 2(C).

MOVEMENT-EVOKED ACTIVITY AFTER INTRA-ARTICULAR INJECTION OF HYADD®4-G AND Hyalgan® IN PMM-TACL KNEE JOINTS

When immediately after PMM-TACL surgery a dose of 100 μ l of HYADD®4-G was injected into the joint cavity the afferent outflow recorded 1 day later was significantly reduced as illustrated by the filled triangles in Fig. 3(A) and the grey bar histogram in Fig. 3(C) ($n = 18$). A comparable result was obtained when HYADD®4-G was given at the time of operation and additionally at day 4, i.e., the third postoperative day, and the neural activity was evaluated 1 week after the initial joint surgery (filled triangles in Fig. 3(B), and white bar histogram, second from left, in Fig. 3(C), $n = 19$). Both outflows are again in the same order of magnitude and significantly below those after PMM-TACL surgery alone. Data are summarized in Table I. Injection of 100 μ l saline instead of HYADD®4-G immediately after surgery and additionally at the third postoperative day also caused a moderate reduction of impulse activity after 1 week compared with untreated PMM-TACL animals (see Table I). Still, the mean reduction of movement-evoked activity 1 week after surgery in PMM-TACL animals treated with HYADD®4-G is significantly larger than in animals injected with saline.

To obtain comparative data with other clinically used elastoviscous materials Hyalgan® was injected immediately after PMM-TACL surgery and at the third postoperative day in another group of experiments. The average movement-evoked afferent outflow recorded 1 week after surgery from 16 fine single afferent is significantly lower than the activity recorded in operated animals without treatment and in animals treated with HYADD®4-G. This result is summarized in Fig. 3(C) (white bar histogram at the right end of the graph) and in Table I.

SPONTANEOUS ACTIVITY

As summarized in Table I, spontaneous impulse activity was present in some fibers of all experimental groups except the group of healthy animals treated with saline and recorded 1 day after injection. Spontaneous activity appeared as occasional impulses fired during the interstimulus periods. The pattern of spontaneous activity in these experimental groups looked like that seen in our previous data¹³. The incidence of spontaneous activity was low (always < 1 Hz) and variable in all experimental groups (17–52%, see Table I).

VARIATION OF KNEE JOINT CIRCUMFERENCE

Confirming previous observations¹³, knee joint diameter increased significantly after surgery both in sham operated and PMM-TACL operated animals (Student's paired t -test. $P < 0.001$). Mean values in the different experimental groups are summarized in Table I.

Discussion

The present results show that the new elastoviscous compound HYADD®4-G has an attenuating effect on

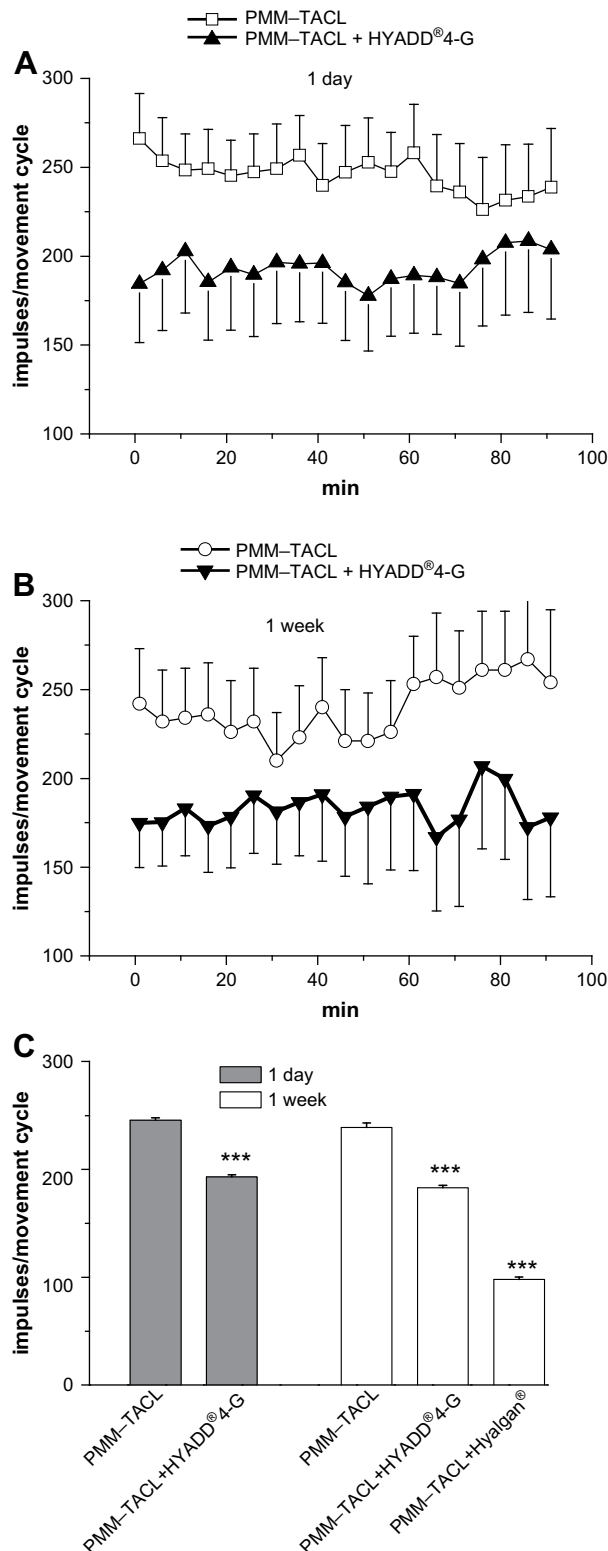


Fig. 3. Movement-evoked activity after PMM-TACL surgery recorded 1 day or 1 week later and having one respectively two post-operative applications of either HYADD®4-G or saline or Hyalgan®. Stimulating, recording, evaluating and plotting as in Fig. 1. (A) Average movement-evoked activity recorded 1 day after surgery in PMM-TACL operated animals (squares; $n = 11$) and in animals which in addition to the PMM-TACL surgery received an injection of HYADD®4-G (solid triangles; $n = 18$) immediately after surgery.

movement-evoked nerve impulse activity from intact knee joints of guinea pigs and also from joints in which an experimental joint injury was induced by partial medial meniscectomy and section of the anterior cruciate ligament. An even more marked attenuation of the activity was also obtained with Hyalgan®, used for viscosupplementation treatment in human OA¹⁹.

Hyalgan® is constituted by 500–730 kDa average molecular weight hyaluronic acid and has a very low elastic modulus¹². HYADD®4-G is equally composed by hyaluronic acid of 500–730 kDa but chemically modified by adding hexadecylamine at a low degree of substitution (2% mol/mol). The manufacturer (Fidia-Farmaceutici) claims that this chemical modification does not cause crosslinking, but the presence of the hexadecylamine determines the formation of additional interactions in the polymer that markedly modifies the rheological properties of the starting hyaluronic acid, thus making this HA derivative more viscous and elastic. Therefore it has been possible in this study to compare the nociceptive effect of two hyaluronic acid products of the same molecular weight with different viscoelasticity²¹.

Our work shows that HYADD®4-G was effective in reducing the excitatory effects of moderate and strong mechanical forces on the impulse activity in fine medial articular nerve afferents of the intact guinea pig knee joint. Similar effects have been described in intact and acutely inflamed rat knee joints with a HA solution of high elastoviscosity, Hyalan G-F 20, while low elastoviscosity materials as Hyalgan® were ineffective¹² thus supporting the interpretation that the elastic and viscous properties of polymer solutions like HA play an important role through their pseudoplastic and molecular shock absorber properties in the intact joint³¹. The elastoviscous behavior reported for HYADD®4-G predicts that this substance also absorbs part of the mechanical energy of the stimulus. This would reduce the transmission of force to the mechanotransduction apparatus at peripheral nociceptive endings, thus decreasing the stimulus force and the response of knee joint sensory fibers to mechanical forces, as was actually observed in the present experiments.

HYADD®4-G and Hyalgan® also reduced significantly the augmented impulse activity seen in operated guinea pigs, 1 day and 1 week after PMM and TACL. In animals with PMM-TACL-induced injury nociceptive discharges evoked by movement remained enhanced only during the early postsurgery period. This suggests that augmented activity is due to a sensitization of joint nociceptors secondary to the inflammatory reaction induced by the surgical trauma, but probably also by cartilage damage that is already observed 1–2 weeks following surgery^{32,33}. The attenuation by HYADD®4-G of this effect cannot be attributed primarily to the elastoviscous buffering action of this substance on mechanical forces transmitted to nociceptive endings

(B) The graphs correspond to those in (A), but the recordings were done 1 week after PMM-TACL surgery. The circles plot the results obtained after surgery alone ($n = 15$), and the triangles give the results obtained when HYADD®4-G ($n = 19$) was applied immediately after surgery and at the third postoperative day. (C) Bar histograms of the results plotted in (A) and (B) as indicated by the lettering. The third white bar at the extreme right shows the average movement-evoked afferent outflow 1 week after PMM-TACL surgery from 16 units where Hyalgan® was injected immediately after surgery and at the third postoperative day. The ** denote the significance for the PMM-TACL operated animals vs those with additional applications as indicated (One-way ANOVA $P < 0.001$).

Table I

Mean values of the number of impulses per movement cycle and number of fibers and animals used in each group are indicated. Also the spontaneous activity recorded in the different experimental groups is shown. The presence of spontaneous activity was considered when the number of impulses per second was higher than 0.05. Last column indicate average of the knee joint circumference measured immediately before surgery and at the day of the recording

Experimental groups			Average number of impulses/movement cycle (S.E.M.)	No. of fibers/No. of animals	Fibers presenting spontaneous activity (imp/s > 0.05)	Joint circumference (cm) Before/After PMM-TACL
1	Healthy animals		211 ± 3	18/16	4/18	—
2	Healthy animals + saline	1 day	191 ± 1.1	10/6	0/10	—
3	Healthy animals + HYADD® 4-G	1 day	73 ± 1	10/5	3/10	—
4	Sham operated	1 day	198 ± 4	12/10	5/12	6.5 ± 0.1/7.5 ± 0.3
		1 week	126 ± 1.5	9/9	2/9	6.5 ± 0.3/7.1 ± 0.2
5	PMM-TACL	1 day	246 ± 2.3	11/7	2/11	6.7 ± 0.2/7.5 ± 0.2
		1 week	239 ± 4	15/10	2/15	6.9 ± 0.2/7.7 ± 0.1
6	PMM-TACL + HYADD® 4-G	1 day	193 ± 2	18/10	3/18	6.3 ± 0.1/7.6 ± 0.2
		1 week	183 ± 2.2	19/9	10/19	6.3 ± 0.1/7.2 ± 0.2
7	PMM-TACL + saline	1 week	218 ± 3.3	8/5	2/8	6.2 ± 0.1/7.5 ± 0.3
8	PMM-TACL + Hyalgan®	1 week	98 ± 2.2	16/10	3/16	6.5 ± 0.3/7.6 ± 0.1

because our work now shows that 1 week after surgery, Hyalgan® was even more effective than HYADD® 4-G in spite of its low elastoviscosity perhaps due to its comparatively higher concentration in HA molecules.

It has been repeatedly suggested that part of the analgesic effect of HA products is due to their binding with the cell membrane receptors and/or chemical interaction with inflammatory mediators released by injured tissues and by inflammatory cells^{1,34–37}. Chemical interaction with inflammatory mediators could explain the marked reduction of impulse activity both with HYADD® 4-G and Hyalgan® 1 week after surgery seen in the present experiments.

Surprisingly, saline injection also attenuated slightly the enhanced activity evoked by surgery, perhaps through dilution of locally released inflammatory mediators. This result could be interpreted as the neurophysiological correlate of the slight but evident reduction in pain experienced by patients with intra-articular injection of placebo or when knee washing is combined with viscosupplementation therapy³⁸.

It is expected that inflammatory agents play a minor role in the modulation of movement-evoked nociceptor activity in intact knee joints but may significantly contribute to its sensitization when inflammation is present as was the case in the present study, judging by the increased knee joint diameter found in operated animals.

The PMM-TACL OA model was aimed at mimicking the symptoms of OA. However, nociceptive discharges evoked by movement in animals after PMM-TACL, were augmented only during the early postsurgery period, possibly reflecting the sensitization of nociceptive afferent units innervating knee joint tissues caused by the inflammatory reaction secondary to surgery. 1 to 2 weeks after PMM-TACL surgery, cartilage damage is evident^{32,33}. Therefore a contribution to joint inflammation of joint instability and altered mechanics cannot be excluded. Nonetheless, the observed changes in impulse activity of knee joint nociceptor fibers in the PMM-TACL OA model do not closely reproduce the characteristics and time course of pain experienced by human patients suffering from OA^{12,13,39}. Thus, extrapolation of the attenuating action on joint nociceptor activity of locally injected HA derivatives in PMM-TACL-induced OA to the analgesic action of these compounds seen in human OA must be made with caution. Taking these limitations into account, it appears reasonable to speculate that HA products have a direct reducing effect on joint nociceptor activity in part due to its role as a mechanical filter that is associated to its

rheological properties but also to chemical interaction with inflammatory mediators present in the inflamed joint tissues, thus reducing their sensitizing effect on nociceptor terminals.

Conflict of interest

None of the authors have any commercial interest associate to the products tested or personal relationships with other people or organisations that could inappropriately influence (bias) their work.

Acknowledgments

We thank E. Quintero and A. Pérez Vegara for technical assistance and to Dr. Antonella Schiavinato for critical reading of the manuscript. This work was supported by funds from FIDIA (Italy), and by grants BFU2005-08741 (CB) and BFU2005-03986 (AG) and CONSOLIDER-INGENIO 2010 CSD2007-00023 to C.B., from the Spanish Ministry of Science and Education.

References

- Balazs EA, Darzynkiewicz Z. The effect of hyaluronic acid on fibroblast, mononuclear phagocytes and lymphocytes. In: Kulonen E, Pikkariainen J, Eds. *Biology of the Fibroblast*. London: Academic Press; 1973:237–52.
- Balazs EA. The physical properties of synovial fluid and the special role of hyaluronan acid. In: Helfet A, Ed. *Disorders of the Knee*. Philadelphia: J.B. Lippincott; 1982:61–74.
- Balazs EA, Denlinger JL. Clinical uses of hyaluronan. *Ciba Found Symp* 1989;143:265–75.
- Balazs EA, Denlinger JL. Viscosupplementation: a new concept in the treatment of osteoarthritis. *J Rheumatol Suppl* 1993;39:3–9.
- Adams ME. Viscosupplementation as articular therapy. Perspective. In: Laurent T, Ed. *The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives*. London: Portland Press; 1996:243–53.
- Weiss C. Hyaluronan and hylan in the treatment of osteoarthritis. In: Kennedy JF, Phillips GO, Williams PA, Hascall VC, Eds. *Hyaluronan*. Cambridge: Woodhead; 2002:467–82.
- Balazs EA. Analgesic effect of elastoviscous hyaluronan solutions and the treatment of arthritic pain. *Cells Tissues Organs* 2003;174:49–62.
- Rydell N, Balazs EA. Effect of intra-articular injection of hyaluronan on the clinical symptoms of osteoarthritis and on granulation tissue formation. *Clin Orthop Relat Res* 1971;80:25–32.
- Peyron JG, Balazs EA. Preliminary clinical assessment of Na-hyaluronate injection into human arthritic joints. *Pathol Biol (Paris)* 1974;22:731–6.
- Pozo MA, Balazs EA, Belmonte C. Reduction of sensory responses to passive movements of inflamed knee joints by hylan, a hyaluronan derivative. *Exp Brain Res* 1997;116:3–9.

11. Pawlak M. Mechanoprotective actions of elastoviscous hylans on articular pain receptors. In: Kennedy JF, Phillips GO, Williams PA, Hascall VC, Eds. *Hyaluronan*. Cambridge: Woodhead; 2002:341–52.
12. Gomis A, Pawlak M, Balazs EA, Schmidt RF, Belmonte C. Effects of different molecular weight elastoviscous hyaluronan solutions on articular nociceptive afferents. *Arthritis Rheum* 2004;50:314–26.
13. Gomis A, Miralles A, Schmidt RF, Belmonte C. Nociceptive nerve activity in an experimental model of knee joint osteoarthritis of the guinea pig: effect of intra-articular hyaluronan application. *Pain* 2007;130:126–36.
14. Belmonte C, Pozo MA, Balazs EA. Modulation by hyaluronan and its derivatives (hylans) of sensory nerve activity signalling articular pain. In: Laurent TC, Ed. *The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives*. London: Portland Press; 1998: 205–17.
15. Just S, Pawlak M, Heppelmann B. Responses of fine primary afferent nerve fibres innervating the rat knee joint to defined torque. *J Neurosci Methods* 2000;103:157–62.
16. Bellamy N, Campbell J, Robinson V, Gee T, Bourne R, Wells G. Viscosupplementation for the treatment of osteoarthritis of the knee. *Cochrane Database Syst Rev* 2006;CD005321.
17. Adams ME, Atkinson MH, Lussier AJ, Schulz JI, Siminovich KA, Wade JP, *et al.* The role of viscosupplementation with hylan G-F 20 (Synvisc) in the treatment of osteoarthritis of the knee: a Canadian multicenter trial comparing hylan G-F 20 alone, hylan G-F 20 with non-steroidal anti-inflammatory drugs (NSAIDs) and NSAIDs alone. *Osteoarthritis Cartilage* 1995;3:213–25.
18. Waddell DD, Cefalu CA, Bricker DC. A second course of hylan G-F 20 for the treatment of osteoarthritic knee pain: 12-month patient follow-up. *J Knee Surg* 2005;18:7–15.
19. Altman RD, Moskowitz R. Intraarticular sodium hyaluronate (Hyalgan) in the treatment of patients with osteoarthritis of the knee: a randomized clinical trial. Hyalgan Study Group. *J Rheumatol* 1998;25:2203–12.
20. Dougados M, Nguyen M, Listrat V, Amor B. High molecular weight sodium hyaluronate (hyalectin) in osteoarthritis of the knee: a 1 year placebo-controlled trial. *Osteoarthritis Cartilage* 1993;1:97–103.
21. Borzacchiello A, Mayol L, Schiavinato A, Ambrosio L. Effect of a hyaluronic acid amide derivative on equine synovial fluid viscoelasticity. *J Biomed Mater Res A (In press)*.
22. Telhag H, Lindberg L. A method for inducing osteoarthritic changes in rabbits' knees. *Clin Orthop Relat Res* 1972;86:214–23.
23. Moskowitz RW, Davis W, Sammarco J, Martens M, Baker J, Mayor M, *et al.* Experimentally induced degenerative joint lesions following partial meniscectomy in the rabbit. *Arthritis Rheum* 1973;16:397–405.
24. Pond MJ, Nuki G. Experimentally-induced osteoarthritis in the dog. *Ann Rheum Dis* 1973 July;32:387–8.
25. Gilbertson EM. Development of periarticular osteophytes in experimentally induced osteoarthritis in the dog. A study using microradiographic, microangiographic, and fluorescent bone-labelling techniques. *Ann Rheum Dis* 1975;34:12–25.
26. Brandt KD, Braunstein EM, Visco DM, O'Connor B, Heck D, Albrecht M. Anterior (cranial) cruciate ligament transection in the dog: a bona fide model of osteoarthritis, not merely of cartilage injury and repair. *J Rheumatol* 1991 March;18:436–46.
27. Karahan S, Kincaid SA, Kammermann JR, Wright JC. Evaluation of the rat stifle joint after transection of the cranial cruciate ligament and partial medial meniscectomy. *Comp Med* 2001;51:504–12.
28. Tessier JJ, Bowyer J, Brownrigg NJ, Peers IS, Westwood FR, Waterton JC, *et al.* Characterisation of the guinea pig model of osteoarthritis by in vivo three-dimensional magnetic resonance imaging. *Osteoarthritis Cartilage* 2003;11:845–53.
29. Anderson-MacKenzie JM, Quasnicka HL, Starr RL, Lewis EJ, Billingham ME, Bailey AJ. Fundamental subchondral bone changes in spontaneous knee osteoarthritis. *Int J Biochem Cell Biol* 2005;37:224–36.
30. Schaible HG, Schmidt RF. Activation of groups III and IV sensory units in medial articular nerve by local mechanical stimulation of knee joint. *J Neurophysiol* 1983;49:35–44.
31. Pena EL, Sala S, Rovira JC, Schmidt RF, Belmonte C. Elastoviscous substances with analgesic effects on joint pain reduce stretch-activated ion channel activity in vitro. *Pain* 2002;99:501–8.
32. Appleton CT, McErlain DD, Pitelka V, Schwartz N, Bernier SM, Henry JL, *et al.* Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. *Arthritis Res Ther* 2007;9:R13.
33. Hayami T, Pickarski M, Zhuo Y, Wesolowski GA, Rodan GA, Duong IT. Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. *Bone* 2006;38:234–43.
34. Gotoh S, Miyazaki K, Onaya J, Sakamoto T, Tokuyasu K, Namiki O. Experimental knee pain model in rats and analgesic effect of sodium hyaluronate (SPH). *Nippon Yakurigaku Zasshi* 1988;92:17–27.
35. Frean SP, Lees P. Effects of polysulfated glycosaminoglycan and hyaluronan on prostaglandin E2 production by cultured equine synoviocytes. *Am J Vet Res* 2000;61:499–505.
36. Akatsuka M, Yamamoto Y, Tobetto K, Yasui T, Ando T. In vitro effects of hyaluronan on prostaglandin E2 induction by interleukin-1 in rabbit articular chondrocytes. *Agents Actions* 1993;38:122–5.
37. Punzi L, Schiavon F, Cavaasin F, Ramonda R, Gambari PF, Todesco S. The influence of intra-articular hyaluronic acid on PGE2 and cAMP of synovial fluid. *Clin Exp Rheumatol* 1989;7:247–50.
38. Vad VB, Bhat AL, Sculco TP, Wickiewicz TL. Management of knee osteoarthritis: knee lavage combined with hylan vs hylan alone. *Arch Phys Med Rehabil* 2003;84:634–7.
39. Denlinger JL. Metabolism of sodium hyaluronate in articular and ocular tissues. Doctoral dissertation. France: Université des Sciences et Techniques de Lille, Lille, 1982, p. 327.